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14. ABSTRACT
Due to its asymptomatic development and frequent diagnosis at advanced stages, ovarian cancer is the most deady among the gynecological cancers. A better understanding of the early molecular events leading to the disease is of utmost importance for the development of strategies for its efficient early diagnosis and prevention, which could improve patient survival and quality of life. We have shown that DMBA-induced mutagenesis in the rat ovary, combined with gonadotropin hormone-mediated enhanced mitogenesis of the ovarian surface epithelium gives rise to lesion s ranging from preneoplastic to early neoplastic and advanced ovarian tumors, which resemble the human disease. The goal of the study is to use this animal model to studay the molecular mechanisms behind ovarian oncognesis and to conduct a preclinical trial for its chemoprevention. The aims of the study are: 1) Determine the molecular genetic mechanisms behind ovarian oncogenesis in the DMBA/gonadotropin-animal model; 2) Determine the efficacy of the COX-1 inhibitor SC-560 to prevent the appearance and/or progression of DMBA-induced ovarian lesions; and 3)Study the in vivo mechanisms of the putative chemopreventive effect of COX-2 inhibition. Genomic and mutation analyses, as well as other molecular biology assay s will be employed to accomplish the objectives of the study.

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Table of Contents

Front Cover 1

SF 298 2

Table of Contents 3

Introduction 4

Body 4

Key Research Accomplishments 9

Reportable Outcomes 10

Conclusions 10

References 10

Appendices ..... N/A

MECHANISMS AND CHEMOPREVENTION OF OVARIAN CARCINOGENESIS

INTRODUCTION

Due to its asymptomatic development and frequent diagnosis at advanced stages, ovarian cancer is the most deadly among the gynecological cancers. A better understanding of the early molecular events leading to the disease is of utmost importance for the development of strategies for its efficient early diagnosis and prevention, which could improve patient survival and quality of life. We have shown that DMBA-induced mutagenesis in the rat ovary, combined with gonadotropin hormone-mediated enhanced mitogenesis of the ovarian surface epithelium gives rise to lesions ranging from preneoplastic to early neoplastic and advanced ovarian tumors, which resemble the human disease. The goal of the study is to use this animal model to study the molecular mechanisms behind ovarian oncogenesis and to conduct a preclinical trial for its chemoprevention. The aims of the study are: 1) Determine the molecular genetic mechanisms behind ovarian oncogenesis in the DMBA/gonadotropin-animal model; 2) Determine the efficacy of the COX-1 inhibitor SC-560 to prevent the appearance and/or progression of DMBA-induced ovarian lesions; and 3) Study the *in vivo* mechanisms of the putative chemopreventive effect of COX-1 inhibition. Genomic and mutation analyses, as well as other molecular biology assays will be employed to accomplish the objectives of the study.

BODY

During the first three years of support by this DoD-CDMRP grant, we have accomplished the following progress along the proposed aims of the study:

1. The goal of specific aim 1 of the study during the first year of support was to generate a large number of DMBA-induced ovarian lesions in the rat at different stages of neoplastic development that would ensure statistical power and significance of the findings from their molecular classification and characterization. Using funds provided by the FCCC NIH-OC-SPORE, in November and December 2003, we initiated a two-phase carcinogenesis experiment in which 160 female Sprague-Dawley rats at 6 weeks of age were subjected to bilateral survival surgery to the ovaries. Animals were separated into four treatment arms and treated: a) Control groups **a1** (20 animals; no hormones) and **a2** (20 animals; with hormones): beeswax-impregnated surgical sutures were implanted in the portion of each ovary that is contralateral to the fallopian tube; b) DMBA-/+hormone group: 100 animals: (**b1**) DMBA/beeswax-impregnated surgical sutures were implanted bilaterally in the ovaries of the animals as above; (**b2**) Two months following the surgical procedure, animals in groups **a2** and **b2** were subjected to 4 cycles of sequential administration of PMSG and hCG. These procedures are described in detail in the Experimental Design and Methods section of our grant proposal and in our recent publication (Stewart et al. *Cancer Res* **64**: 8177-83, 2004). All treated animals were maintained for 12-13 months from the survival surgical procedure, or until disease development and animal distress became apparent. Animals were sacrificed according to the initiation of treatment, in December

2004 and January 2005, following guidelines approved by the FCCC IACUC committee, the NIH and the DoD-CDMRP.

The ovaries of all animals were harvested and fixed in 70% ethanol at 4°C for 18hr, following which they were paraffin processed through a 12-hour cycle with a Tissue-Tek VIP 5 (Sakura) vacuum infiltration processor, and then paraffin embedded with a Histo-Center II (Fischer Scientific) embedding station. Three 5μm-thick sections, app. 50μm apart of each other were obtained from the two end-portions of each ovary, stained with H&E and subjected to histopathology examination to determine the presence of ovarian lesions.

Table 1 below indicates the incidence of lesions observed in the four experimental arms, subdivided into 3 subgroups (non-neoplastic, putative precursors and neoplastic). This experiment was conducted in order to determine the potential promoting role of gonadotropin hormones in ovarian cancer development, and to generate sufficient numbers of ovarian lesions for molecular characterization and determining the mechanisms behind their development. Based on the observed, statistically significant differences in lesion incidence between arms **a1** and **a2**, and **b1** and **b2** (Table 2), we conclude that gonadotropin hormones play a major role in the promotion of ovarian preneoplasia and neoplasia.

Table 1. DMBA ovarian carcinogenesis with gonadotropin co-treatment

Experimental Arm	*per ovary*				*per animal*			
	No Lesions	Non-Neoplastic Lesions	Putative Pre-Neoplastic Lesions	Neoplastic Lesions	No Lesions	Non-Neoplastic Lesions	Putative Pre-Neoplastic Lesions	Neoplastic Lesions
a1 - Surgery only (20 animals) %	37.5	40.0	22.5	0.0	0.0	70.0	30.0	0.0
a2 - Surgery+Hormones (19 animals) %	20.8	21.1	58.1	0.0	0.0	26.1	73.9	0.0
b1 - DMBA (47 animals) %	15.7	20.5	62.8	1.0	6.3	13.0	78.7	2.1
b2 - DMBA+Hormones (45 animals) %	1.1	15.4	75.8	7.7	0.0	8.8	75.8	15.4

Table 2. Statistical significance of differences in lesion incidence induced by gonadotropin co-treatment (* - determined by χ-square and/or Fisher's exact tests)

Comparison*	Site of the lesions	P-value
Surgery vs. Surgery+Hormones	Ovary	0.0061
	Animal	0.0064
DMBA vs. DMBA+Hormones	Ovary	0.0002
	Animal	0.0422

Based on the histopathological characteristics and stage of neoplastic development, ovarian lesions detected in **b2** ovaries were subdivided into the following 7 categories: 1) normal OSE cells (>35 samples); 2) reti ovarii hyperplasia (12 lesions); 3) bursal and OSE flat hyperplasia (28 lesions); 4) bursal and OSE papillary hyperplasia (34 lesions); 5) inclusion serous cyst with papillary hyperplasia (40 lesions); 7) non-invasive carcinoma (3 lesions); 7) invasive adenocarcinoma (5 lesions). OSE cells appearing normal and obtained from ovaries of **a1** (>15 samples) and **a2** (>15 samples) animals generated 2 additional sample (control) categories.

Molecular characterization of DMBA-induced ovarian lesions

To determine whether, similar to the human disease, COX-1 and/or COX-2 expression/activation is associated with ovarian neoplastic development in this model, we initiated a collaboration with Dr. S.K. Dey at Vanderbilt University Medical Center. Microscopic slides were prepared from tissue sections obtained from formalin-fixed paraffin-embedded rat ovaries treated with DMBA or DMBA/hormones and containing putative preneoplastic (7 samples) or neoplastic lesions (5 samples). Each slide also contained a tissue section from the corresponding contra-lateral, control ovary. Individual slides, sent to Dr. Dey, were subjected to immunohistochemical analysis for COX-1 or COX-2 expression. Elevated expression of both enzymes was observed in the majority of analyzed putative preneoplastic lesions and all neoplastic lesions regardless of progression. Neither protein was detectable in the OSE of normal (control) ovaries. Although in most cases, the expression level of COX-1 was higher than that of COX-2, the data suggests a strong association of both enzymes with ovarian cancer development in this model. Examples of COX-1/2 changes in expression are shown in Figure 1. These results are quite interesting, and although support our original proposal for the

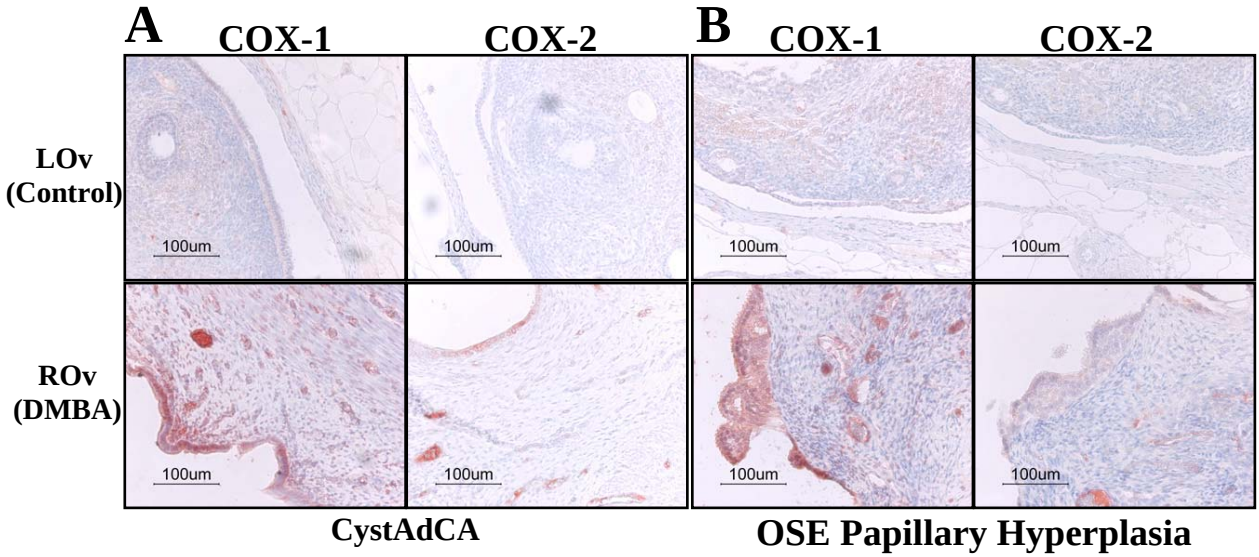

Figure 1. IHC staining for COX-1 (left half-panel A and B) and COX-2 (right half-panel A and B) protein expression in rat ovaries: Left (LOv) untreated control (top panels) and Right (ROv) DMBA-treated (lower panels). **A.** Cystadenocarcinoma; **B.** Surface epithelial papillary hyperplasia. Sections of LOv and ROv from same animal were mounted on same microslide and subjected to IHC at identical conditions. Pictures of each pair of sections per microslide were taken at identical brightness/contrast settings.

pre-clinical testing of a COX-2 specific inhibitor (celecoxib) (please see **2.** below), they also suggest that a COX-1 specific inhibitor (such as SC-560, Cayman Chemical Co) may be more efficacious as an agent for chemoprevention of ovarian cancer. The results also warrant further analysis of additional ovarian lesions, both putative preneoplastic and neoplastic, in order to evaluate the prevalence of the observed changes in COX-1/2 expression, and whether they are

also present in putative preneoplastic lesions induced by gonadotropin hormone treatment alone. In addition to IHC analysis, *in situ* hybridization may be also used as an alternative approach on slides generated from ethanol-fixed ovarian tissue specimens.

We have also conducted a global, microarray-based gene expression analysis of multiple human ovarian tumors and normal human ovarian surface epithelia (non-cultured or short-term cultured). Among the genes identified with differential expression between different types of tumors and normal OSE, the most interesting was the NF-κB regulator gene A20. While this gene was found expressed at moderate to high levels in the normal OSE, its expression was undetectable in all tested tumors, regardless of their histological subtype or neoplastic stage

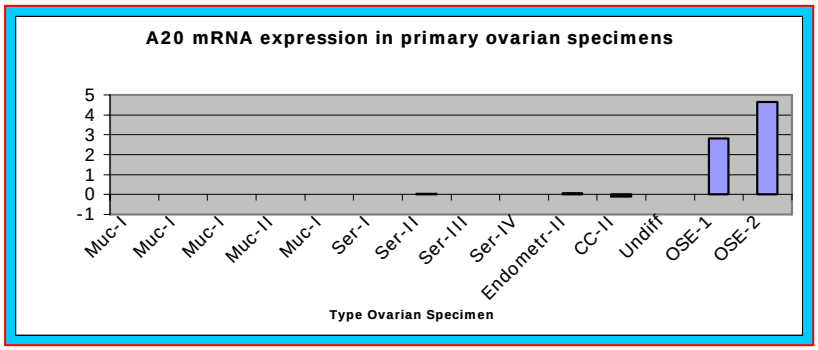

Figure 2. Microarray-determined A20 mRNA expression in primary human ovarian cancer specimens of different histological subtype and malignant stage, and in normal human OSE (OSE-1: average of 4 short-term cultures; OSE-2: average of 2 non-cultured samples). Data was confirmed by real-time qRT-PCR analysis (not shown)

(Fig. 2). This result suggests that A20 plays a confounding role in the development of ovarian carcinomas and could potentially play such a role also in the DMBA-induced ovarian carcinogenesis. A20 is an enzyme with dual ubiquitination and de-ubiquitination activities and plays an essential role as a switch between activation and inactivation of the NF-κB survival transcription factor. While A20 facilitates the coupling of cytokine and other receptor signals to the IKK signalosome complex through RIP and other MAP3Ks, it is also essential for termination of the same signals and inhibition of a persistent NF-κB activation. The persistent, elevated activation of NF-κB has been associated with the malignant progression and development of resistance to cytotoxic treatment of many types of tumors. Hence, loss of A20 in ovarian cancer may be one of the mechanisms underlying this process and may be a very important target for the design of new strategies for prevention and treatment of the disease. In support of this conclusion are the encouraging preliminary results obtained from a phase I trial of the proteasome inhibitor bortezomib in combination with platinum agents (carboplatin) for overcoming the development of chemoresistance of ovarian cancer patients.

We have initiated a study to investigate the expression status of A20 in the normal rat OSE and in DMBA-induced lesions at different stages of neoplasia. This study is based on real-time qRT-PCR analysis of RNA purified from corresponding rat ovarian samples, selected by LCM, and amplified as described below. Guided by our microarray validation studies for human ovarian samples, rat A20 specific oligonucleotides have been designed and the conditions for reproducible RT-PCR amplification of the gene will be optimized for real-time qRT-PCR analysis. This will be accomplished by determining the linear range of amplification of A20 in different RNA preparations purified from normal OSE obtained from control untreated and gonadotropin-treated animals. Based on our results from the analysis of human normal OSE, we expect that A20 is also expressed at moderate levels in the normal rat OSE.

Genomic analysis of DMBA-induced rat ovarian lesions

As indicated in our earlier annual report, for the molecular classification and analysis of the obtained ovarian lesions, we have decided to utilize the Affymetrix GeneChip system, instead of the originally proposed rat oligonucleotide microarrays produced at the FCCC DNA microarray

facility. Instead of investing funds and effort in generating rat oligo-arrays of inconsistent quality at the FCCC microarray facility, we decided to use the Affymetrix platform, which now has available the Rat 230 2.0 GeneChip arrays. These are comprised of more than 31,000 probe sets, analyzing over 30,000 transcripts and variants from over 28,000 well-substantiated rat genes. The Human Genetics Research Program at FCCC has purchased the Affymetrix GeneChip system that includes the GeneChip Fluidics Station450 for automated microarray washes, the GeneChip Scanner 3000 for microarray image capture, and the GeneChip Operating Software (GCOS) V.1.2 for microarray image and data analysis.

We have evaluated which is the best experimental protocol for the amplification of total RNA purified from cells selected from individual ovarian lesions using laser-capture microdissection (LCM). For this purpose, we used ovaries of control rats (not included in above carcinogenesis experiment) that were harvested, fixed and embedded as above. Four to six 5μm-thick sections were generated, deparaffinized, rehydrated, stained with HistoGene LCM Frozen Staining Kit (Arcturus), and 2,000-5,000 OSE cells were collected on CapSure HS LCM Caps using an AutoPix Automated LCM apparatus (Arcturus) and a laser-beam diameter of 10μm ± 2 μm (power: 50-70mW; pulse: 2200-2800μsec; intensity: 200mV)using a diameter of 7-10μm. Total RNA was immediately isolated from the microdissected cells using the PicoPure RNA Isolation Kit (Arcturus), yielding ~5ng of total RNA. RNA quantification and integrity assessment were carried out by microfluidic electrophoresis on a 2100 Bioanalyzer using the RNA 6000 Pico Chip LabChip Kit (Agilent Technologies).

Total RNA has been subjected to parallel amplification using a) an Ovation Aminoallyl RNA Amplification and Labeling System (NuGen Technologies) and b) a BioArray High Yield RNA Transcript Labeling Kit (ENZO). The product from a) is an anti-sense aminoallyl-substituted cDNA, which can be used for both oligonucleotide and cDNA microarray gene expression analysis, as well as for real-time qRT-PCR-based verification of the microarray results. We wanted also to evaluate the product of b), which although represents a biotin-labeled anti-sense aRNA, it has been used successfully by other research groups in conjunction with Affymetrix microarray chips, and because it is considerably more cost-effective than the Ovation amplification kit. After 2 rounds of RNA amplification, the quality of the resulting amplified RNA products from a) and b) was evaluated by real-time qRT-PCR using oligonucleotides specific for genes known to be expressed at high, moderate and low levels (GAPDH, p53, and Tpl-2, at a relative average expression ratio of app. 5,000:500:30 respectively; this information was obtained from the Rat Ovarian Gene Expression Database: http://app.mc.uky.edu/kolab/rogedendo.aspx). Our results indicated a very similar amplification quality by both kits, so for the purposes of genomic analysis of the DMBA-induced ovarian lesions we will use the ENZO BioArray amplification kit.

A number of groups, both from academia and industry, have reported that a considerable variation in the microarray data is incorporated when different sets of arrays are used to compare specimens in a single experiment. This is an issue that may have a significant impact on the reproducibility and reliability of the microarray data generated for the purpose of this research. To avoid this, and since the preparation of tissue specimens, purification and amplification of RNA and quality testing are the rate-limiting procedures, we decided to first process all lesion samples to the point that we could carry out all microarray hybridizations serially within a short period of time and with the same lot of GeneChip arrays.

As described in our previous report, we have achieved a complete histopathology examination of all 262 ovaries harvested from the 131 animals included in the 4 arms of the

carcinogenesis experiment described above. This allowed the identification of ovaries that contain different types of lesions and the selection of lesions for the purpose of this study according to their classification, as illustrated above. In a stream-lined fashion, ovaries selected for a certain type of lesion have been subjected to further processing in preparation for genomic analysis. Depending on the size of lesion and its epithelial cell component, 4-6 5μm-thick sections were generated from the portion of the organ adjacent to the corresponding H&E-stained sections and processed for LCM, as described above. Alternatively, following deparaffinization, dehydrated sections were stored at –80°C until they were used for LCM. Immediately prior to LCM, sections were rehydrated, stained with HistoGene LCM Frozen Staining Kit (Arcturus), and subjected to LCM as described above to select epithelial cell component (2,000-5,000 cells) from corresponding lesions or normal OSE. Total RNA was obtained from all microdissected samples and subjected to quantification and integrity assessment as described above. Total RNA samples with acceptable quality were then subjected to the first round of amplification using the ENZO BioArray amplification kit. In the first round of amplification, bacteriophage T7 RNA polymerase promoter sequence is incorporated into the synthesized first cDNA strand. Next, exogenous primers are added to the second-strand reaction in order to make ds-cDNA. In-vitro transcription is then performed using T7 RNA polymerase. After the first round of amplification, purified aRNA is converted into ds-cDNA, which is the template for a second round of amplification. However, for purposes of stability during storage and quality assessment, the resulting ds-cDNAs were stored at -20°C until further use. Currently, all ds-cDNAs generated from the first round of RNA amplification have been subjected to a second quantification and integrity assessment using the 2100 Bioanalyzer (Agilent), as well as by quantitative real-time PCR with GAPDH oligonucleotides performed on a SmartCycler (Cepheid) thermal cycler. Approximately 1/15-th volume of resulting ds-cDNA is used for this purpose at a 200x dilution. A good-to-excellent quality cDNA product usually yields a single, distinct melting peak with a linear Ct value within the range of 18-25 cycles.

During the first 3 months of the third year of support we completed the quality assessment of all cDNAs generated from amplified lesion-RNAs. However, due to the pending transfer of the PI of this grant to Temple University, no other considerable progress has been made along the lines of the proposed research. Similarly, the planned chemoprevention trial experiment was not initiated, as it was postponed until after the transfer of the PI to Temple University. Unfortunately, the PI has had no access to laboratory facilities between July 31 and August 31 and after his transfer to Temple University between September 1, 2006 and February 28, 2007. Consequently, no further progress has been made along the lines of the proposed research.

Due to his pending transfer to the NCI, NIH, the PI of this grant has requested that this grant is transferred to a new PI at FCCC. Dr. Dusica Cvetkovic has agreed to continue the research proposed by this grant during the last year of funding by the DoD. Dr. Cvetkovic is a member of the Ovarian Cancer Research Group at FCCC and has the necessary credentials and expertise for conducting the proposed research. All samples and reagents generated during the first three years of support by this grant have been transferred to her. The PI, Dr. Christos Patriotis will provide Dr. Cvetkovic with all necessary assistance for the successful completion of the proposed research.

KEY RESEARCH ACCOMPLISHMENTS

No considerable progress has been made along the lines of the proposed research due to the pending and consequent transfer of the PI to Temple University, and the lack of

laboratory facilities during the first 6 months (September 1, 2006 through February 28, 2007) of his appointment there.

REPORTABLE OUTCOMES

None

CONCLUSIONS

We have shown that administration of gonadotropin hormones in rats leads to the development of ovarian epithelial lesions of putative preneoplastic nature that resemble those appearing in ovaries of animals exposed to DMBA alone or DMBA/gonadotropins. This result, as well as the observed statistically significant increase in ovarian tumor incidence and malignant progression in animals treated with DMBA/gonadotropin versus DMBA alone, direct application of a low dose of DMBA conclusively demonstrate that gonadotropin hormones promote ovarian cancer development. We have also shown that the protein expression of COX-1, and to a lesser degree COX-2, is significantly increased in putative preneoplastic and neoplastic ovarian lesions induced by DMBA or DMBA/gonadotropins. Recently, our microarray-based genomic analysis of primary human ovarian cancer specimens revealed that the expression of the dual ubiquitin-editing enzyme A20, a key regulator of NF-κB activation, is lost during ovarian cancer development. This conclusion is based on the fact that A20 mRNA expression, which is detected at a moderate level in normal human OSE cells (cultured or not), is below reliably detectable levels in all ovarian tumor specimens tested, regardless of histological subtype or stage of malignancy. Hence, loss of A20 may represent an early, confounding event in ovarian oncogenesis, and may be associated with the frequently observed increased, persistent activation of NF-κB, and potentially with the development of resistance to platinum-based chemotherapy. The expression of A20 in DMBA-induced ovarian lesions at different neoplastic stages will be investigated. If a similar status of A20 is determined, it will be very exciting to examine the molecular mechanisms leading to loss of its expression and its potential relevance to the prevention and therapy of ovarian cancer.

REFERENCES

None